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CITATION:

KAJIHARA, Hiroshi. A histology-free description of the branched-proboscis ribbonworm Gorgonorhynchus albocinctus sp. nov. (Nemertea: Heteronemertea). Publications of the Seto Marine Biological Laboratory 2015, 43: 92-102

ISSUE DATE:

2015-09-15

URL:

http://hdl.handle.net/2433/199852

Publ. Seto Mar. Biol. Lab., 43: 92–102, 2015

A histology-free description of the branched-proboscis ribbonworm *Gorgonorhynchus albocinctus* sp. nov. (Nemertea: Heteronemertea)

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Abstract A new species of heteronemertean with branched proboscis, *Gorgonorhynchus albocinctus* sp. nov., is described based on the external feature and DNA sequences from a specimen collected subtidally among coral rubbles at a depth of about 6 m in a reef area around Taketomi Island in the Yaeyama Islands, southwestern Japan. It can be classified in the genus because the mode of proboscis branching is dichotomous, rather than alternating laterally. From the two known congeners *G. repens* Dakin and Fordham, 1931 and *G. bermudensis* Wheeler, 1940, the new species can be differentiated by having a brown body colour with a white band near the tip of the head. The new species is placed in a molecular phylogenetic context with a maximum-likelihood analysis using the nuclear 18S rRNA and 28S RNA genes, as well as the mitochondrial cytochrome c oxidase subunit I gene, together with 20 species of heteronemerteans, for which those sequences were available in the public databases. The results indicate that *G. albocinctus* is closely related to *Cerebratulus leucopsis* (Coe, 1901), a species that is supposed to have a non-branched proboscis.

Keywords: Pilidiophora, Gorgonorhynchidae, COI, DNA barcoding, East China Sea, Philippine Sea, marine invertebrates

Introduction

Six species in five genera of heteronemerteans have been known to possess a branched proboscis, in contrast to the normal, straight, tubular organ found in the rest of the members in the phylum. These are *Gorgonorhynchus repens* Dakin and Fordham, 1931, *G. bermudensis* Wheeler, 1940, *Panorhynchus argentinensis* Serna de Esteban and Moretto, 1969, *Polybrachiorhynchus dayi* Gibson, 1977, *Polydendrorhynchus zhanjiangensis* (Yin and Zeng, 1984), and *Oligodendrorhynchus hesperides* Fernández-Álvarez and Anadón, 2012. The species in *Gorgonorhynchus* Dakin and Fordham, 1931 are different from others in that the proboscis is dichotomously branched, whereas in others it is comprised of a main stem with alternating lateral branches that can be further forked into tertiary or quaternary branchlets (Gibson, 1977; Sun, 2006; Fernández-Álvarez and Anadón, 2012). The two congeners, *G. repens* and *G. bermudensis*, are both distributed in warmer waters, with the former having been reported from eastern Australia (Dakin and Fordham, 1931; Gibson, 1981), Bikini Atoll (Coe, 1947), and India (Graveley, 1927); and the latter from Bermuda (Wheeler, 1940) and Florida (Gibson, 1974).

A cladistic analysis of heteronemerteans by Schwartz and Norenburg (2001), based on internal morphological characters extracted and coded from literature, resulted in a fully unresolved tree, indicating that internal anatomy—at least those characters that have traditionally been mentioned in taxonomic

descriptions—is uninformative for the purpose of phylogeny inference. When establishing the heteronemertean *Pseudomicrura afzelii* Strand and Sundberg, 2011, these authors proposed a new standard in nemertean species description. It is based on external features and DNA sequences, without examination of internal anatomy by serial histological sections, a methodology that has been traditionally employed in nemertean systematics (e.g., Gibson, 1985). Strand and Sundberg (2011) advocated that description of internal morphology is not only useless in inferring phylogeny (at least within certain subgroups of the phylum, e.g. Heteronemertea) but also of little help in species identification. In a way, Strand and Sundberg (2011) appear to coincide with a recently-proposed approach called "turbo- taxonomy" (Butcher et al., 2012; Riedel et al., 2013) that aims fast species descriptions combining cyto- chrome c oxidase subunit I (COI) gene sequences, concise morphological descriptions, and high-resolution digital imaging.

In this paper, I describe a new species of branched-proboscis heteronemertean in the genus *Gorgonorhynchus,* adopting the approach that Strand and Sundberg (2011) put forward, based on its DNA sequences and a concise description of external features accompanied with digital images, which are necessary and sufficient for species identification and systematization. With the DNA sequences obtained, I place the new species in the context of molecular phylogeny using other heteronemertean sequences that are available in the public databases.

Material and Methods

A single specimen was collected subtidally among coral rubbles at a depth of 6 m during SCUBA diving in a reef area around Taketomi Island, 24°17.95′ N, 124°05.21′ E, on 16 November 2013 by Junko Inoue. It was photographed alive, anaesthetized by dropwise addition of 35 psu $MgCl_2$ solution; its posterior tip was cut off and preserved in 99% EtOH, while the rest was placed in 10% formalin–seawater; these processes were executed by Shinri Tomioka. The type material has been deposited in the Hokkaido University Museum, Sapporo, Japan (catalogued under the acronym ZIHU).

Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, USA) from the tissue fragment. PCR amplification of the nuclear 18S and 28S rRNA genes, as well as the mitochondrial COI gene were performed with the following primer pairs: for 18S, 1F / 9R (Giribet et al., 1996); for 28S, LSU5 (Littlewood, 1994) / 3KR (Yamasaki et al., 2013); and for COI, LCO1490 / HCO2198 (Folmer et al., 1994). Thermal cycling condition was 95°C for 1 min, 35 cycles of 95°C for 30 sec, 45°C for 90 sec, and 72°C for 3 min (90 sec for COI), then 72°C for 7 min. Sequences were determined using the BigDye Terminator Kit ver. 3.1 (Life Technologies Co., USA) and a 3730 DNA analyzer (Life Technologies Co., USA), using, in addition to the same primer pairs used for PCR amplification, the following internal primers: for 18S, 2F, 4F, 6R, and 8R (Giribet et al., 1996); and for 28S, Sa (Whiting et al., 1997), rd4b (Edgecombe and Giribet, 2006), rd4.8a (Schwendinger and Giribet, 2005), rd5b (Schwendinger and Giribet, 2005), F2012 (Giribet et al., 2010), and rd7b1 (Schwendinger and Giribet, 2005). Base calling and assembling were performed with ATGC ver. 4.0.6 (Genetyx).

To assess the phylogenetic affinities of the new species, a preliminary analysis was carried out with 20 species of heteronemerteans, for which COI, 18S, and 28S sequences were available in the public databases (Table 1). Outgroups were chosen to include *Baseodiscus mexicanus* (Bürger, 1893) and *B. unicolor* (Stiasny-Wijnhoff, 1925), since *Baseodiscus* has been shown to be sister to 'Schizonemertini' (i.e., heteronemerteans with lateral cephalic slits) in recent molecular phylogenetic analyses (cf. Andrade et al., 2012; Kvist et al., 2014). Sequence alignment was carried out gene by gene with MUSCLE (Edgar, 2004a, b) implemented in MEGA ver. 5.2.1 (Tamura et al., 2011), then concatenated in Kakusan4 ver. 4.0 (Tanabe, 2011); no significant nucleotide compositional heterogeneity was detected for the combined data set ($p = 1.00000$ by chi-square test in Kakusan4). After eliminating gap positions, the dataset comprised 531 nt (COI),

1623 nt (18S), and 1805 nt (28S). A shot-gun maximum-likelihood (ML) analysis was performed with RAxML ver. 8.0.0 (Stamatakis, 2014) under general time-reversible model (Tavaré, 1986) considering discrete gamma distribution for among-site rate variation, based on parameters optimized by Kakusan4 with four categories of gamma rate, starting with a tree generated by neighbour-joining method (Saitou and Nei, 1987) using Jukes and Cantor's (1969) distance. The shot-gun search was executed with 10 inferences on the original alignment using 10 distinct randomized maximum-parsimony tree. Bootstrap support values were calculated using 1000 pseudoreplicates.

Table 1. Taxa included in the phylogenetic analysis, with accession numbers in the public databases.

Taxa	Markers		
	18S	28S	COI
Ingroup (Lineidae)			
Cerebratulus lacteus (Leidy, 1851)	JF293044[a]	HQ856857[a]	KC698905[b]
Cerebratulus leucopsis (Coe, 1901)	KF935300[c]	KF935356[c]	KF935517[c]
Gorgonorhynchus albocinctus sp. nov.	LC010650[d]	LC010651[d]	LC010649[d]
Lineus acutifrons Southern, 1913	JF304778[a]	HQ856855[a]	GU590937[e]
Lineus bilineatus (Renier, 1804)	JF293041[a]	HQ856844[a]	GU392015[f]
Lineus torquatus Coe, 1901	JF293035[a]	HQ856856[a]	EF124972[g]
Lineus viridis (Müller, 1774)	JF293032[a]	HQ856854[a]	KC812597[h]
Micrura chlorapardalis Schwartz and Norenburg, 2005	KF935292[c]	KF935348[c]	KF935512[c]
Micrura fasciolata Ehrenberg, 1828	JF293039[a]	HQ856847[a]	GU392022[f]
Micrura ignea Schwartz and Norenburg, 2005	JF293043[a]	KF935342[c]	KF935507[c]
Micrura purpurea (Dalyell, 1853)	JF293036[a]	HQ856845[a]	HQ848586[a]
Micrura verrilli Coe, 1901	KF935288[c]	KF935344[c]	KF935508[c]
Notospermus geniculatus (Delle Chiaje, 1828)	KF935295[c]	KF935351[c]	EF125002[g]
Notospermus sp. 1	KF935296[c]	KF935352[c]	KF935515[c]
Notospermus sp. 2	KF935298[c]	KF935354[c]	KF935516[c]
Parborlasia corrugata (McIntosh, 1876)	JF293037[a]	HQ856851[a]	KC812593[h]
Ramphogordius lacteus Rathke, 1843	JF293065[a]	KF935358[c]	KF935519[c]
Ramphogordius sanguineus (Rathke, 1799)	JF293040[a]	HQ856853[a]	AJ436938[i]
Riseriellus occultus Rogers et al., 1993	JF293033[a]	HQ856849[a]	DQ911397[j]
Outgroup (Valenciniidae)			
Baseodiscus mexicanus (Bürger, 1893)	KF935281[c]	KF935337[c]	KF935503[c]
Baseodiscus unicolor (Stiasny-Wijnhoff, 1925)	KF935285[c]	KF935341[c]	KF935505[c]

Sources: [a]Andrade et al. (2012); [b]Lu et al. (unpubl.); [c]Kvist et al. (2014); [d]present study; [e]Puerta et al. (2010); [f]Strand and Sundberg (2011); [g]Schwartz and Norenburg (unpubl.); [h]Strand et al. (2014); [i]Thollesson and Norenburg (2003); [j]Sundberg and Strand (2007).

Results and Discussion

Taxonomy

Gorgonorhynchus albocinctus sp. nov.
(Figs. 1–2)

http://zoobank.org/:pub:99CB19F0-392A-42EF-91E9-8B852903068B

Material examined. ZIHU 4976, anterior fragment of body and detached proboscis, preserved in 10% formalin–seawater.

Sequences. Newly determined sequences from the holotype have been deposited in DDBJ with the following accession numbers: LC010649, COI (658 nt); LC010650, 18S rRNA (1792 nt); and LC010651, 28S rRNA (2096 nt).

Description. Holotype lacking tail when collected (Fig. 1A); presence / absence of caudal cirrus unknown. Anterior fragment of body 6 cm in length, up to 6 mm in width. In life, dorsally brown, ventrally paler, with white band encircling slightly posterior to tip of head; colour faded and becoming greyish in preserved material, but the white subterminal band still recognizable (Fig. 1B–D). Tip of head darker, almost black in colour (Fig. 1A), obtuse from dorsal view, not rectangular as in *Notospermus*. Head not demarcated from succeeding portion of body. Horizontal lateral cephalic slits extending back to anterior level of mouth (Fig. 1D). Eyes not found. Anterior portion of body rather rounded cross-sectionally, gradually flattened posteriorly; in intestinal region, lateral margin of body edged as in *Cerebratulus*.

Proboscis partially everted, detached from body while fixation (Fig. 1E), white in colour. Due to incomplete state of evertion, exact branching pattern difficult to interpret, but appearing to be more or less dichotomous (Fig. 2A), with 32 branches (Fig. 2B).

Remarks. Apparently, the anaesthetization was not sufficiently in effect when the animal was fixed, as the proboscis was everted and detached from the body, but this made the specimen identified as the member of *Gorgonorhynchus*. The new species differs from the two congeners by the body colour, being brown with a white subterminal band *vs*. bright reddish-orange in *G. repens*, light pink or red overall in *G. bermudensis*; neither of the latter two have any white band (Gibson, 1974). In the Northwest Pacific, three species of lineids have been reported to possess a similar white subterminal band near the tip of the head: *Cerebratulus insignis* Punnett, 1900 from Singapore, *Cerebratulus albocirculus* Iwata, 1957 from Sagami Bay, Japan, and *Lineus albovittatus* (Stimpson, 1855) from Okinawa, Japan.

In *C. insignis,* the body has an olive-green colour and the tip of the snout is almost black (Punnett, 1900); the latter characteristics is also true to some extent in *G. albocinctus* (Fig. 1A). The subterminal white band in the preserved specimen of *C. insignis* (Punnett, 1900, pl. 6, fig. 14) is depicted as broad as that of *G. albocinctus* (Fig. 1B–D). According to Punnett (1900), the proboscis was missing in the type material of *C. insignis*; therefore, the possibility cannot be ruled out that the species actually has a branched proboscis, being closely related to *G. albocinctus*.

Cerebratulus albocirculus was described based on preserved material, and its living state is unknown. Iwata (1957) did not mention any peculiarity about the proboscis in *C. albocirculus*, thus the proboscis in this species is likely to be a normal, non-branching type, having less phylogenetic affinity to *Gorgonorhynchus*.

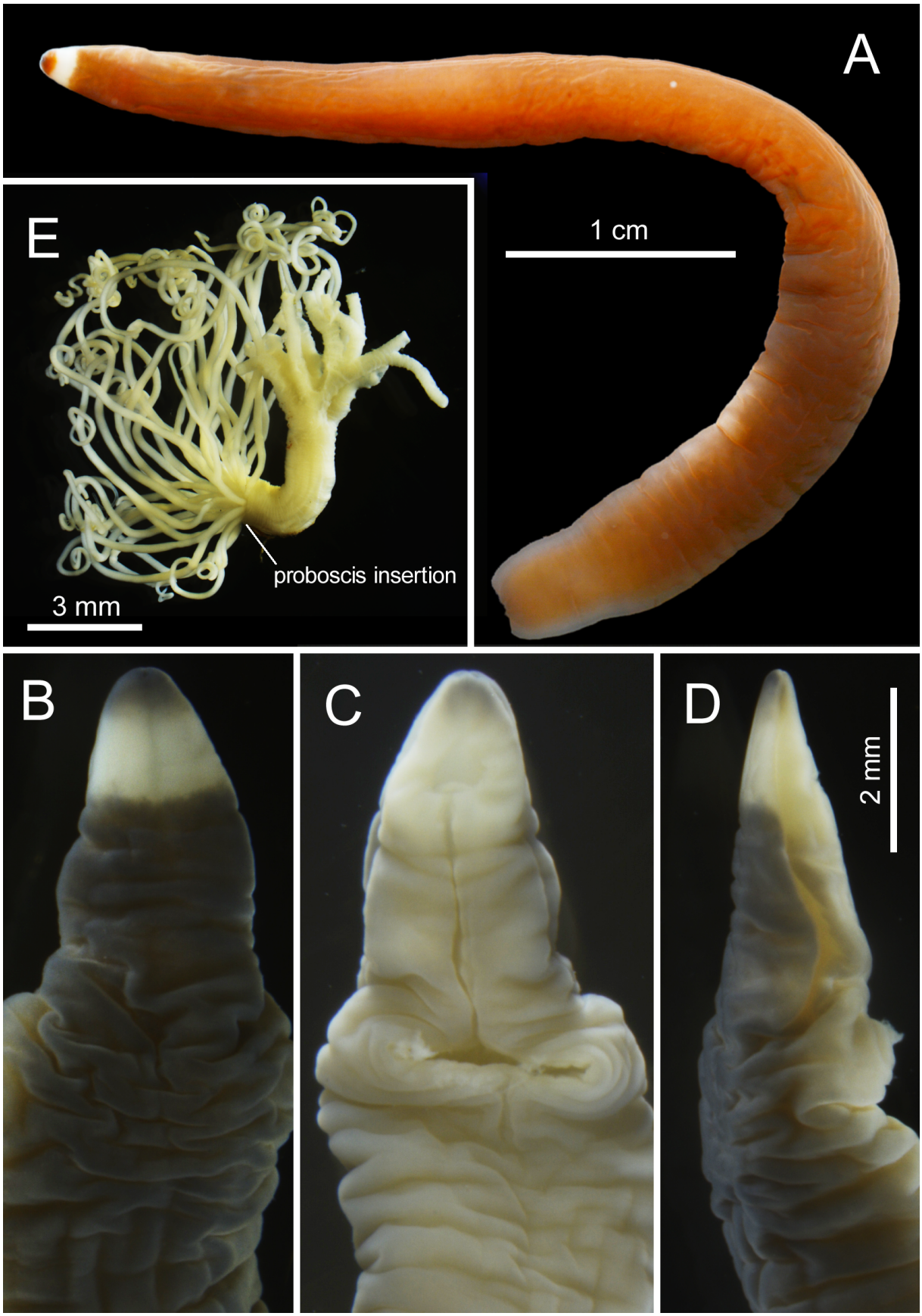

Figure 1. ***Gorgonorhynchus albocinctus*** sp. nov., holotype, ZIHU 4976. A, photograph taken in life by Shinri Tomioka; B–E, preserved material. A, anterior fragment, dorsal view; B, head, dorsal view; C, head, ventral view; D, head, right lateral view; E, proboscis, incompletely everted and detached from body.

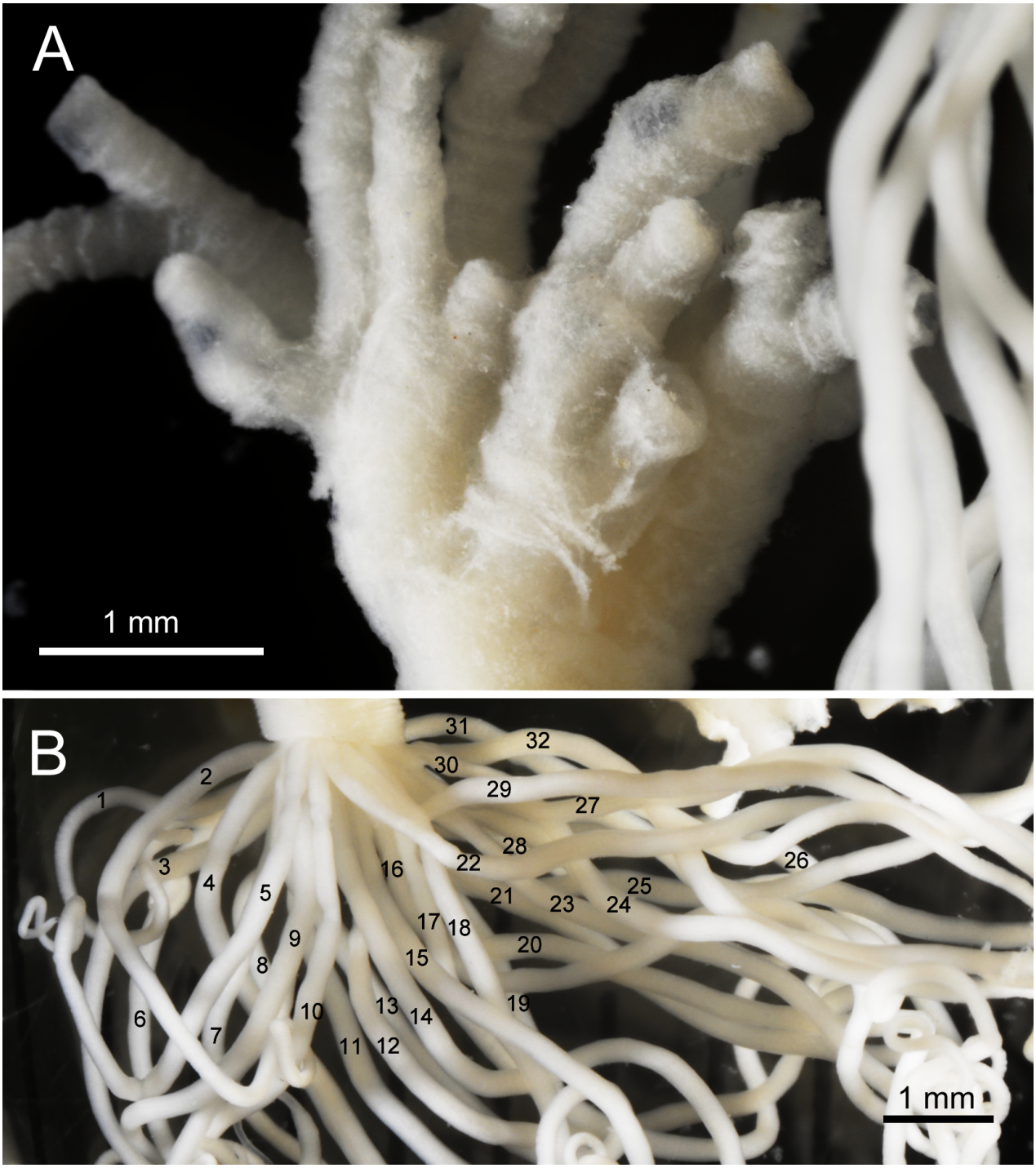

Figure 2. ***Gorgonorhynchus albocinctus*** sp. nov., holotype, ZIHU 4976, detached proboscis in preserved state. A, partially everted portion, showing the branching pattern; B, retracted portion, showing 32 branches.

The fixed specimen of *C. albocirculus* was yellowish green in colour, with the dark green head having a narrow white transverse ring (Iwata, 1957, pl. 1, fig. 7a–c), which appears to agree with *Lineus albovittatus* that is reported to have a grass-green body with the head "crossed by a white band at a point about the middle of the lateral fissures" (Stimpson, 1855: 382). Altogether, these green forms may closely related to *Lineus fuscoviridis* Takakura, 1898 that has uniformly green body, and possibly also to *Notospermus geniculatus*

(Delle Chiaje, 1828) that shows variably green to brown body with numerous white bands along the body. On the other hand, *L. albovittatus sensu* Iwata (1954) has white basement body colour with broad, velvet-green longitudinal dorsal stripe, thus appearing to represent a different taxon from *L. albovittatus s.str*.

A BLAST search (Altschul et al., 1997) at the NCBI website (http://blast.ncbi.nlm.nih.gov) with the COI sequence of *G. albocinctus* (LC010649) showed that it was 85% identical with those of *Micrura dellechiajei* (Kvist et al., 2014) (with 99% query coverage) and '*Dendrorhynchus sinensis*' [the name should be *Polydendrorhynchus zhanjiangensis*: see Kajihara et al. (2008) for nomenclatural discussion)] (Xu, unpublished, direct submission) (with 93% query coverage), suggesting a close phylogenetic relation between them.

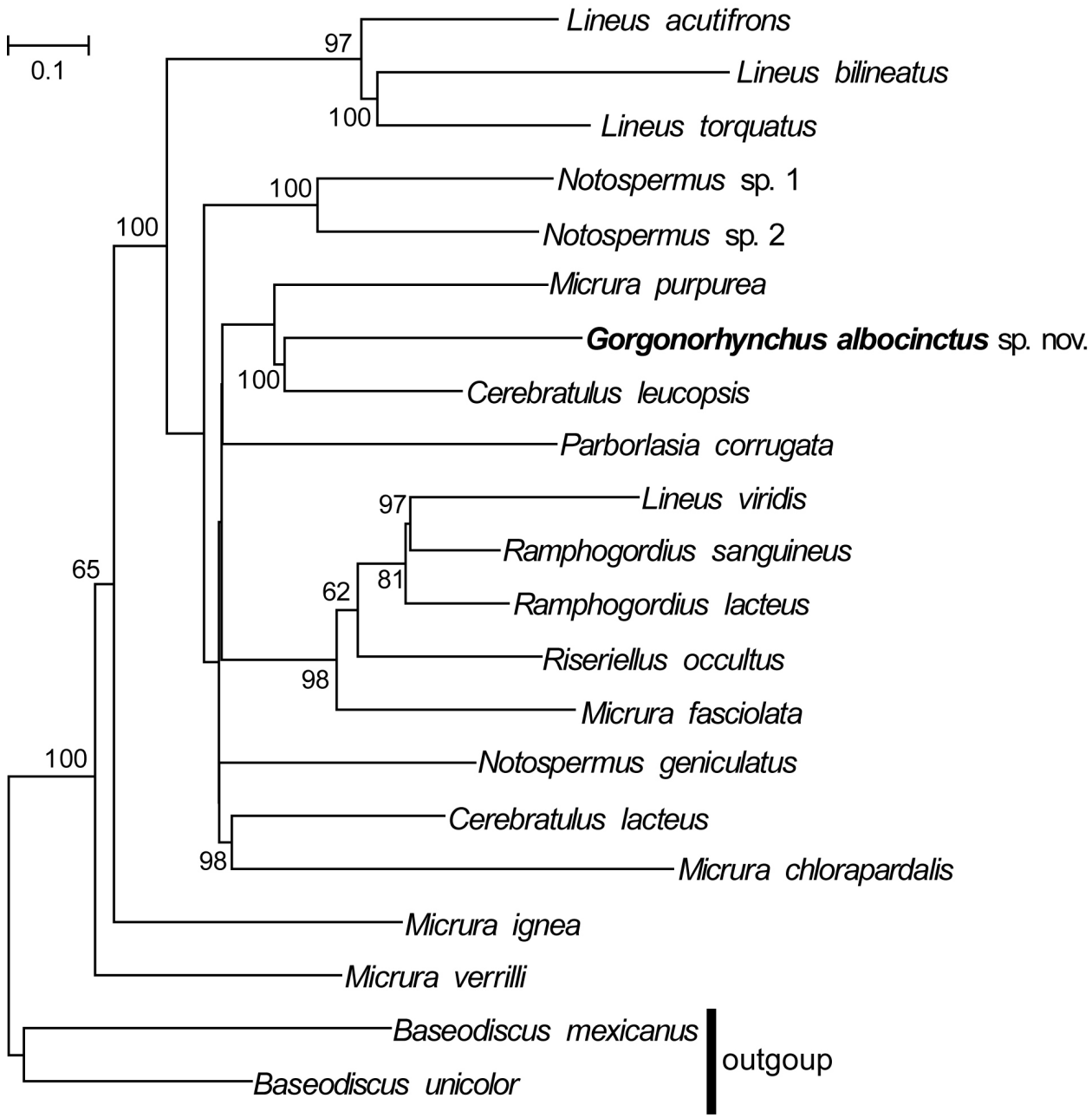

Figure 3. Phylogenetic tree resulting from maximum-likelihood analysis of combined COI, 18S, and 28S rDNA sequences (ln L = −19143.329006). Numbers near nodes indicate bootstrap support values ≥ 50%.

Molecular Phylogeny

In the resulting maximum-likelihood tree (ln L = −19143.329006) (Fig. 3), *G. albocinctus* was the sister taxon to *Cerebratulus leucopsis* (Coe, 1901), supported with 100% bootstrap frequency (BF), although the latter species is supposed to have a non-branched proboscis. The tree topology is thus in favour of Chernyshev's (2011) synonymization of Gorgonorhynchidae with Lineidae. Other well-supported clades recovered in the analysis include: *Lineus acutifrons* Southern, 1913 + *L. bilineatus* (Renier, 1804) + *L. torquatus* Coe, 1901 (97% BF); *Notospermus* sp. 1 + sp. 2 (100% BF); *L. viridis* (Müller, 1774) + *Rampho-gordius sanguineus* (Rathke, 1799) + *Ra. lacteus* Rathke, 1843 + *Riseriellus occultus* Rogers et al., 1993 + *Micrura fasciolata* (Dalyell, 1853) (98% BF); and *C. lacteus* (Leidy, 1851) + *M. chlorapardalis* Schwartz and Norenburg, 2005 (98% BF). As has been repeatedly pointed out in previous studies (Sundberg and Saur, 1998; Andrade et al., 2012; Kvist et al., 2014), most of the heteronemertean genera as currently diagnosed are non-monophyletic. Ideally, future analyses including as many type species of genus-group names as possible should be done for proper application of genus names for many species.

Acknowledgments

I am grateful to Kazumi Okuda and Yoshimichi Akutsu (dive shop Ishigaki Sensui Dou) for their cooperation in the field survey; to Daisuke Uyeno (Kagoshima University) and Hiroshi Yamasaki (University of Ryukyus) for organizing the field survey; to Junko Inoue (University of Tokyo) and Shinri Tomioka (Hokkaido University) for collecting and processing the specimen; to Shushi Abukawa (Hokkaido University) for help in molecular work; to Shimpei Hiruta (Hokkaido University) and Keiichi Kakui (Hokkaido University) for assisting phylogenetic analysis; and to Malin Strand (Swedish University of Agricultural Sciences) and Alexei V. Chernyshev (A.V. Zhirmunsky Institute of Marine Biology) for their valuable comments on the manuscript. This study was financially supported by JSPS KAKENHI Grant Numbers 23370038 (Principal Investigator: Matthew H. Dick) and 2565013 (Principal Investigator: Hiroshi Kajihara).

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Received: 7 Apr 2015
Accepted: 15 May 2015
Published: 15 Sept 2015